Peer Review File
Article information: http://dx.doi.org/10.21037/atm-20-3661

Reviewer A

1. This paper investigates the effects of a new decellularization protocol in the enthesis. The effect of this VAS process is evaluated using a holistic approach to show that it more efficiently and more successfully removes cells while retaining the structural, mechanical, and biological benefits of the tissue. I believe that the paper is well organized and interesting.

Reply: Sincere thanks for your comments and encouragements to our study.

2. For the histomorphological analysis you mention that the samples were sectioned but it is unclear if these were frozen or parafinized sections. I believe these are deminerlized sections but it is not mentioned. Please clarify.

Reply: Sincere thanks for your comments.

Owing to our inappropriate expressions, we are sorry for giving you such confusion. In fact, the C-AEM, O-AEM and native enthesis tissue (NET) were demineralized and then sectioned for histological staining. In the revised manuscript, we modified it to make our expression more clearly. Please see the revised manuscript. (see Page 7, line 157-161)

3. For the DNA analysis you do not mention how the tissue was broken down. Freeze and then use a dismembranator?

Reply: Sincere thanks for your comments.

Owing to our inappropriate expressions, we are sorry for giving you such confusion. We should describe the procedure more detail. In fact, the freeze-dried samples were weighed and minced with tissue grinder. In the revised manuscript, we modified it to make our expression more clearly. Please see the revised manuscript. (see Page 7, line 165-169)

4. For the water absorption study, what is your starting condition? Id the starting weight the lyophilized weight?

Reply: Sincere thanks for your comments.

Owing to our inappropriate expressions, we are sorry for giving you such confusion. In fact, we weighted the freeze-dried NET, C-AEM and O-AEM firstly as M1, and then immersed them into PBS for 24-hour and weighted again as M2. Absorption was calculated according to the following equation: absorption (%) = (M2-M1)/M1 × 100%.

6. Why did you decide to test the entheses dry? Wouldn't it be significantly more relevant to test them after rehydration?

Reply: Sincere thanks for your comments.

During SEM and EDS, the specimens must be dried to meet the requirements of testing equipment. As for mechanical test, we need to ensure that the cross-section area of NET, C-AEM and O-AEM at the enthesis interface is equal, thus ensure that there is no error resulting from the size of sample during tensile test. The rehydrated NET, C-AEM and O-AEM specimens is soft, no convenient for trimming the specimens into the similar cross-section area at the enthesis interface. (see Page 8, line 202-204)

7. You claim that you have removed all of the cells, but you also mention that there is still some DNA and some DAPI signaling. Doesn't that indicate that this isn't a 100% successful process.

Reply: Sincere thanks for your comments.

In fact, the DAPI signaling in the C-AEM and O-AEM specimens was induced by overexposure, which was not produced from the residual DNA. We chose overexposure to make the figure more vivid. In the revised manuscript, we modified the figure 2.

In addition, the DNA content in the C-AEM and O-AEM specimens were lower than 50 ng/mg, which is in line with the standard of decellularization. Moreover, HE staining images showed no cell nucleus.

8. In Fig 7A, does the red represent mineralized and the green unmineralized? Does that mean that your fibrocartilage region has one mineralized point and one unmineralized data point? In the previous figure you use the red, green and blue for bone/fibrocartilage/tendon. I recommend that you stay consistent with your colors across the whole paper to make it clearer to the reader.

Reply: Sincere thanks for your comments.

Sorry for our carelessness. In fact, we should use the red, green and blue to represent bone, fibrocartilage, tendon, respectively. In the revised manuscript, we corrected it. Please see the revised **Figure 7**.

9. In figure 4 your points in the tendon region are different colors. Are they meant to represent different things?

Reply: Sincere thanks for your comments.

Owing to our inappropriate expressions, we are sorry for giving you such confusion. In fact, the different colors in the tendon region were set automatically when drawing with Prism 8.0 software. In the revised manuscript, we corrected it.

Please see the revised **Figure 4**.

10. The grammar/vocabulary gets worse as the paper progresses. Things like saying that the samples were weighted instead of weighed. Please have the grammar reviewed. Especially in the discussion.

Reply: Sincere thanks for your comments.

We are sorry for these grammatical and spelling errors, as well as incorrect scientific terms. As your suggested, we asked a help from a commercial language polishing company to edit our manuscript. Please see the revised manuscript.

Reviewer B

1. The authors present a new bone-plug-based approach to tissue engineered repair of the rotator cuff enthesis. The approach is a new and useful take on an old and lingering problem. The reviewer is enthusiastic.

Reply: Sincere thanks for your comments and encouragements to our study.

2. The big picture of the enthesis. The authors cite at the beginning of the paper the old fashioned four-zone model of the enthesis, and proceed in their study to look for features of this. This reviewer (who is a strong proponent of the functional gradient model) feels that this is outdated and incorrect. However, the reviewer recognizes that others still believe in the four-zone model and will not oppose publication of a paper based upon the latter.

Nevertheless, the reviewer feels that the authors have ample evidence in their data showing that their approach succeeds in producing a clean, graded, repair of the enthesis, and asks them to consider the following points.

Reply: Sincere thanks for your comments and encouragements to our study.

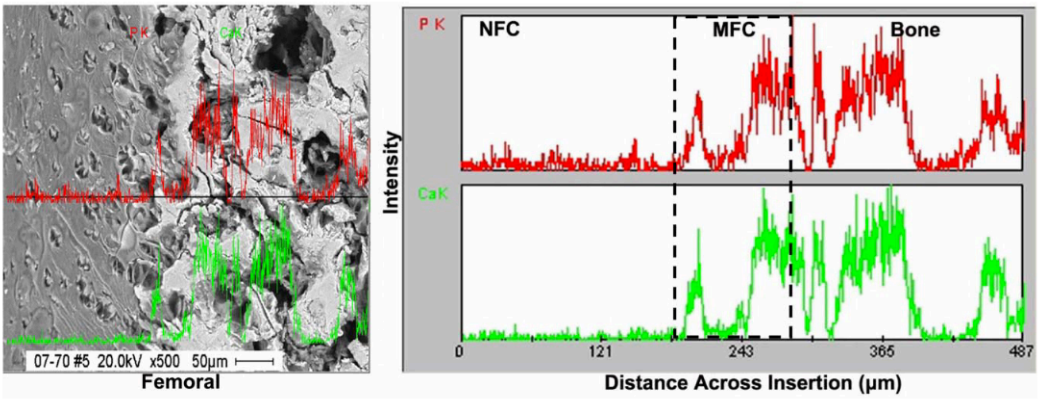
Indeed, the four-zone model of the enthesis is old fashioned, and more and more researchers think the enthesis is transitional, gradient structure. In fact, our study is not focused on four-zone model or functional gradient model. we just want to introduce a way to decellularize a large-sized enthesis tissue, and histologically determine the in-vivo performance of decellularized enthesis matrix on rotator cuff enthesis regeneration. In the revised manuscript, we modified our expression to avoid this controversial issue (four-zone model or functional gradient model). (Line 69, 453)

3. The authors' data seem to show that their method retains a linear gradient in mineralization, which has been shown to reduce stress concentrations (relevant papers follow; these include work by the reviewer that the authors should not feel compelled to cite: https://pubmed.ncbi.nlm.nih.gov/28541313/,

https://pubmed.ncbi.nlm.nih.gov/28250445/, https://pubmed.ncbi.nlm.nih.gov/19686644/). The region of graded mineral appears to be intact using the authors' approach, but not attached using the old approach, and important advance. If the authors are willing to torture their graduate students, quantifying this would make for a nice addition to the paper (or would be a nice follow-on paper); however, it might suffice simply to mention this if the authors are in agreement.

Reply: Sincere thanks for your comments.

Indeed, adding more data about the graded mineral distribution will improve the quality of our manuscript. According to our experience and published literature[1], SEM+EDS can only show the mineral distribution in the form like the following figure, in which the graded mineral distribution in the enthesis cannot be vividly presented.



In our previous study[2], synchrotron radiation-based micro X-ray fluorescence (SR-μXRF) analysis is a suitable way to present this graded mineral distribution in the enthesis. However, recently, our group have not authority to use this technique in Shanghai Synchrotron Radiation Facility, China. Thus, we just used SEM+EDS to evaluate the Ca and P in the bony, fibrocartilage, and tendinous region of enthesis. Our results indicated that the Ca and P in the NET were well preserved after decellularization with our or conventional protocol.

4. Other key features of the functional gradient model are the orientation distribution of collagen (a paper that the authors should not feel compelled to cite, but that highlights the importance of this: https://pubmed.ncbi.nlm.nih.gov/24352669/) and the distribution of non-collagenous proteins (a paper that highlights the importance of this; again no need to cite: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5761353/). The authors seem to have exceeded the state of the art in producing smooth and appropriate gradients of these. If the authors are as excited about this as the reviewer, it might make sense to consider quantifying and/or discussing this.

Reply: Sincere thanks for your comments.

In the revised manuscript, we added some sentences to discuss the point you mentioned in the Discussion part. Please see the revised manuscript. (see Page 21, line 505-506)

5. Engineering metrics of performance. The reviewer feels that toughness is a key component of enthesis function. From the strength and stiffness results reported, one cannot tell whether the new technology produces a more resilient enthesis or simply a strong, stiff, but brittle one. For example, glass is very stiff and strong, but not very effective at absorbing energy. Given the images shown in the paper, the functional gradient model of attachment would predict that the toughness of the authors' repairs would greatly exceed those of the state of the art. This can be estimated from the area under the force-displacement curves for the failure tests. Would the authors consider adding those?

Reply: Sincere thanks for your comments and suggestion.

Precisely speaking, we should add some parameters about toughness of regenerated enthesis in this study. But considering the following factors, we finally used the histological staining for postoperative evaluation only. Firstly, the aim of this study is to introduce a novel acellular protocol for large-size enthesis, and we evaluated the fabricated large-size acellular enthesis matrix from the aspect of physicochemical and biological properties. The in-vivo function is an aspect of the fabricated AEM. But not the most critical. The critical point is focused on the preservation of ECM components and tensile resistance. In this study, we just want to elucidate the efficacy of AEM on regenerating bone-fibrocartilage-tendon structure in-vivo, the histological staining is enough for evaluating this. While the mechanical test is a whole function index used for measure the regenerated enthesis, cannot elucidate the AEM on regenerating bone-fibrocartilage-tendon structure. In some cases, the regenerated enthesis showed superior tensile property, while did not regenerate a typical bone-fibrocartilage-tendon structure histologically. Secondly, if we added the tensile test into our study, the sample size of canine will be enlarged, thus double research funding, even more. To avoid the concern as you mentioned, this problem was discussed in the limitations of Discussion part. (see Page 22, line 541-544)

6. The name of the protocol. The authors have not actually optimized anything, and the reviewer feels that the name "O-AEM" is not likely to catch on as a consequence. How about something like "Direct Removal Enthesis Acellular Matrix" for DREAM or something similar? Then the old-fashioned approach could be "Chemical Removal Enthesis Cellular Matrix" (CREAM, which in colloquial English means to destroy).

Reply: Sincere thanks for your comments and suggestion.

In the revised manuscript, we modified the name of O-AEM and C-AEM according to your meaningful suggestion. This expression may avoid your concern. Please see the revised manuscript.

7. How about a catchier title for the paper, optimized to get attention in the literature? The reviewer is worried that this very nice contribution might not receive the attention it deserves with the current title. Maybe something like "Direct removal of cells produces clinically relevant scaffolds for improved enthesis repair" (Or "Vacuum removal ...." or "Detergent free..."). Or perhaps simply "Improved acellular scaffolds for enthesis repair"?

Reply: Sincere thanks for your comments and suggestion.

We changed the title for this paper into “Designing a Novel Vacuum Aspiration System to Decellularize Large-size Acellular Enthesis with Preservation of Physicochemical and Biological Properties”.

8. Last sentences of the introduction and conclusions. Are these perhaps over-stated? Would it make sense to change "This optimized protocol can be applied to efficiently decellularize large-size enthesis as scaffolds for augmenting enthesis regeneration in clinic." to something more like "The protocol shows promise for augmenting enthesis regeneration in clinic."?

Reply: Sincere thanks for your comments and suggestion.

In the revised manuscript, we modified the last sentences of the introduction and conclusions according to your suggestion.

9. Colors in the graphs. The reviewer would have appreciated having colors in the graphs have the same meaning throughout (for example, O-AEM to be the same color everywhere in the paper).

Reply: Sincere thanks for your comments and suggestion.

In the revised manuscript, we used the black color to represent the NET, the red color to represent the C-AEM, the green color to represent the O-AEM.

10. In the methods, the authors do not note the animal source of the entheses. Did they obtain canine shoulders from a slaughterhouse? Please clarify the sentence in the methods.

Reply: Sincere thanks for your comments.

In fact, canine enthesis specimens with bony attachment were harvested from dogs sacrificed in local slaughterhouse. (see Page 6, line 133)

References

[1] K. L. Moffat, W. H. Sun, P. E. Pena, N. O. Chahine, S. B. Doty, G. A. Ateshian, C. T. Hung, H. H. Lu, Proceedings of the National Academy of Sciences of the United States of America 2008, 105, 7947.

[2] H. Lu, C. Chen, Z. Wang, J. Qu, D. Xu, T. Wu, Y. Cao, J. Zhou, C. Zheng, J. Hu, Spectrochimica Acta Part B: Atomic Spectroscopy 2015, 111, 15.